Journal of Surgical Oncology 62:97–108 (1996)

Significance of Spontaneous Apoptosis During Colorectal Tumorigenesis

MASAHIKO KOIKE, MD
From the First Department of Pathology, Aichi Medical University, Aichi, Japan

To determine if apoptosis is involved in colorectal tumorigenesis and its progression, colorectal adenomas (n = 63), carcinomas (n = 49), and normal mucosa were investigated by using in situ end-labeling (TUNEL) method. The expression of Ki-67 was also analyzed immunohistochemically. TUNEL labeling index (TLI) and Ki-67 labeling index (KLI) were determined. TLI/KLI was significantly higher in the adenomas of small size and/or of low and middle grade atypia than those of large size and/or of high grade atypia. No difference was observed in the indices between adenomas and carcinomas and among the cancer groups classified on the basis of their clinicopathological features. The results indicate that the reduction of susceptibility to apoptosis plays an important role in the early stage of the adenoma-carcinoma sequence. Apoptosis can explain the enormous cell loss thought to exist in normal colorectal mucosa and in the tumor growth process.

KEY WORDS: **colorectal cancer, colorectal adenoma, nick end labeling method, Ki-67, p53, bcl-2**

INTRODUCTION

Apoptosis, a mode of cell death in which single cells are deleted in the midst of tissues, accounts for the many kinds of cell death observed in living tissues (e.g., most of the programmed cell death during development and the physiological cell death in the course of normal tissue turnover) [1–3]. Even in tumors, apoptosis occurs widely, although it is not the only mode of death adopted by tumors. Irradiation, chemotherapy, and the appropriate hormone therapy all induce apoptosis in tumor cells [4]. Without those stimuli, apoptosis occurs spontaneously [4]. Some regulatory genes that influence the cellular susceptibility to apoptosis have been identified and are already familiar as oncogenes and oncosuppressor genes [5–10]. Apoptosis, therefore, is supposed to play an important role in carcinogenesis and its progression.

Various aspects related to apoptosis have been investigated intensively. There are, however, few morphological studies investigating the distribution and frequency of apoptosis in human solid tumors during their development and progression. The reason is that precise and quantitative observation of the cells in the process of apoptosis is difficult in the specimens stained by routine histological techniques such as hematoxylin and eosin (H/E) staining. The morphological changes of the apoptotic cells, such as shrunken and fragmented cytoplasm with condensed nuclear chromatin, are certainly characteristic, but: (1) an apoptotic process is a very swift and effective means to remove dying cells from tissues without leaving traces and thus cells at an early stage of apoptosis may escape detection, (2) the morphological changes are not always apparent enough to be a sure marker of apoptotic cells in situ, and (3) nonapoptotic cells with condensed chromatin (e.g., mitotic cells or neutrophils) may be confused with apoptotic cells.

The initiation of the apoptotic process is known to be associated almost exclusively with the internucleosomal cleavage of DNA [11]. Gavrieli et al. [12] have developed a new method to detect DNA fragmentation by using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL). In this method, TdT specifically binds labeled dUTP to 3′-OH ends of

Accepted for publication January 23, 1996.

Address reprint requests to Masahiko Koike, M.D., First Department of Pathology, Aichi Medical University, 21 Karimata Yazako Nagakute-cho Aichi-gun, Aichi 480-11, Japan.

DNA and the cells with DNA fragmentation can be visualized in situ.

The purpose of this study is to show the role of apoptosis in the process of tumorigenesis and the progression in vivo. Colorectal adenomas have been identified as the precursor lesion of colorectal adenocarcinomas and provide an ideal model to study tumor progression. Some cumulative genetic alterations are involved in this tumor progression [13]. In order to determine if the apoptosis is involved in the colorectal adenoma-carcinoma sequence, the apoptotic cells were identified in normal colon mucosa, colorectal adenomas, and carcinomas using the TUNEL method. The frequency and distribution pattern of apoptotic cells were analyzed quantitatively with those of cells expressing Ki-67, bcl-2, and p53 by the immunostaining method in the same specimens.

MATERIALS AND METHODS

Patients and Tissue Samples

In this study, 63 adenomas and 49 cancers, including seven cases of carcinoma in adenoma, were obtained from 43 patients who underwent colorectal surgery without preoperative adjuvant therapy, e.g., chemotherapy or radiation, and 29 patients who had endoscopic polypectomy for colorectal polyps at the First Department of Surgery (Aichi Medical University). Normal mucosa on the same section was used for the normal control. Materials from the specimen were fixed in 10% formalin for <24 hours and were embedded in paraffin. Serial sections (3 μm) were cut and affixed to glass slides coated with poly L-lysin.

Detection of Apoptosis-Associated DNA Fragmentation In Situ by the TUNEL Method

DNA fragmentation in situ associated with apoptosis was detected by TUNEL as described by Gavrieli et al. [12]. Sections were deparaffinized, washed with distilled water (DW), treated with proteinase K (20 mg/ml) in 10 mM Tris-HCL buffer (pH 7.4) incubated at room temperature (RT) for 20 minutes, washed with DW, and incubated with 2% aqueous H_2O_2 solution for 5 minutes to eliminate endogenous peroxidase. The sections were washed with DW and immersed in TdT buffer (30 mM Trizma base, 140 mM sodium cacodylate, 1 mM cobalt chloride, pH 7.2). TdT (Takara Shuzo Co., Japan, 0.3 e.u./ml) and biotinylated dUTP (Boehringer Mannheim/Yamanouchi, Japan, 10 mM) in TdT buffer were added to cover the sections and then incubated at 37°C for 90 minutes in a humidified chamber. The sections were washed with TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 minutes, washed with PBS, covered with 2% aqueous solution of BSA for 10 minutes, and washed again with PBS. The sections were then covered with streptavidin conjugated with peroxidase for 30 minutes in RT, washed in PBS, and stained with 3-3′-diaminobenzidine tetrahydrochloride (DAB). For the positive control, thymus glands of 4-week-old mice excised 4 hours after irradiation with 6Gy were used.

Immunohistochemical Staining for Ki-67, bcl-2, and p53

Paraffin sections to be stained for Ki-67, bcl-2, and p53 were pretreated by microwave irradiation. In brief, after being deparaffinized and rehydrated, the sections were placed in stainless steel containers that were submerged to the bottom of a glass beaker filled with 0.01 M citrate buffer (0.01 M citric acid, 0.01 M sodium citrate), irradiated, and boiled five times for 5 minutes in a microwave processor at 600w power output. Thereafter the sections were allowed to cool down to room temperature. Anti-Ki-67 (clone MIB-1, Immunotec, France), anti-bcl-2 (clone 124, DAKO, Denmark), and anti-p53 (clone 1801, Oncogene Science) were used as primary antibodies at a dilution of 1:100. The sections were covered with normal horse serum for 10 minutes, incubated with primary antibody at RT for 40 minutes, and washed with PBS. Sections were then immersed in cold methanol with 0.3% aqueous H_2O_2 for 15 minutes, washed with PBS, incubated with biotinylated anti-mouse IgG for 30 minutes, and washed again with PBS. Next, the sections were reacted with streptavidin conjugated with peroxidase for 10 minutes, washed with PBS, and finally stained with DAB. For positive controls, infiltrating lymphocytes were used for bcl-2 and proliferating cells in the crypts of normal colon mucosa were used for Ki-67. A specimen of colon cancer with positive expression of p53 in our laboratory were used for positive control of p53.

Double Staining by a Combination of the TUNEL Method and Ki-67 Immunostaining

Ten sections of cancer and five of adenoma were stained by combination of the TUNEL method and the immunohistochemical method for Ki-67. In brief, after being reacted with MIB-1 for the primary antibody and antimouse IgG conjugated with horseradish peroxidase (Tago) for the second antibody, the sections were stained for horseradish peroxidase with 3-amino-9-methylcarbazole (AEC). Then the TUNEL method was applied. To avoid cross-reaction, streptavidin conjugated with alkaline phosphatase was substituted for streptavidin labeled with peroxidase. Fast blue was used as a chromogen.

Statistical Methods

More than four microscopic fields randomly selected were photographed in all sections stained by TUNEL and immunohistochemistry for Ki-67. In each picture, 1,000 or more tumor cells were counted, and the ratio of the positive cells to all counted cells was determined. In each section, a mean value was calculated and the TUNEL

labeling index (TLI, TUNEL positive cells/total cells counted) and Ki-67 labeling index (KLI, Ki-67 positive cells/total cells counted) were determined. In order to analyze the kinetics of the apoptosis and the proliferation among various kinds of tumors, the ratio of TLI/KLI, which meant the ratio of the apoptotic cells to the tumor cells in the growth fraction, was calculated. In the analysis of the preparations stained with anti-bcl-2 and anti-p53 antibodies, when >30% of the specimen was stained positive, the preparation was considered positive. All data were presented as the mean ± standard error. Student's *t*-test and the Pearson rank correlation test were used for analysis.

RESULTS

Distribution of TUNEL and Ki-67 Positive Cells in Colorectal Cancers

In colorectal carcinoma, Ki-67 positive cells and TUNEL positive cells were randomly distributed (Fig. 1). Comparing the arrangement of the TUNEL positive cells with that of Ki-67, positive cells were located close to one another in the same cancer tubules, but showed no particular relationship to each other. Among the TUNEL positive cells, apoptotic bodies predominated, but some cells with normal-looking nuclei also intermingled. In mirror sections stained with TUNEL and H/E (Fig. 2), tumor cells in various stages were observed from the stage of normal looking to that of apoptotic bodies. In some cases, many TUNEL positive cells were seen in the lumen of the cancer tubules. Apparently, some of them were apoptotic bodies, but the other cells were indistinguishable from inflammatory cells or degenerated cancer cells (Fig. 1). Thus, TUNEL positive cells were counted only among the cancer epithelial cells in the statistical analysis. The average TUNEL- and Ki-67 labeling indices were 2.83% and 33.1%, respectively (Table I).

Double Staining Consisting of TUNEL and Ki-67 Immunostaining of Tumors

In the specimens stained by a combination of TUNEL and Ki-67 immunostaining, the apoptotic cells took two staining patterns: (1) cells positive with TUNEL alone, and (2) cells positive with both TUNEL and Ki-67 (Fig. 3).

Distribution of TUNEL and Ki-67 Positive Cells in Normal Colon Mucosa

In the normal mucosa, Ki-67 positive cells were mostly observed in the lower half of the crypt, defined as the proliferation zone, and no positive cells were seen on the surface of the crypt (Fig. 4). The Ki-67 labeling index in the lower half of the normal crypt was 15.7 ± 1.9% (Table I). On the contrary, TUNEL positive cells were observed in two parts of the crypts; the uppermost part at the edge of the crypt facing the gut lumen and the lower half of the crypt (Fig. 4). In the upper crypt area, several TUNEL positive cells formed clusters in some of the crypts. A few neighboring crypts with stained cells and a few neighboring crypts without these existed in turn. The morphology of the stained cells in the cluster was not different from that of unstained cells and never showed apoptotic bodies (Fig. 4). The TUNEL positive cells in lower crypts, however, existed sporadically and were the typical apoptotic bodies (Fig. 4). The frequency of TUNEL positive cells in the lower crypts was much lower than in the upper parts. One positive cell was observed in every 5–10 crypts on average. Since TUNEL positive cells in normal mucosa were few and there were many crypts without the positive cells, the labeling index was difficult to calculate precisely. It was estimated to be <0.5% on average (Table I).

Distribution of TUNEL and Ki-67 Positive Cells in Colorectal Adenomas

Ki-67 labeled cells were observed along the whole length of the tubules of adenoma corresponding to the normal crypt (Fig. 5). Proliferative activity expanded and spread over the surface epithelium. TUNEL positive cells were also observed along the whole length of the adenoma tubules (Fig. 5), but the staining pattern at the uppermost part was different from that in the other part of the crypt. In the uppermost part of the tubules of adenoma corresponding to the normal crypt, the TUNEL positive cells had the same pattern as in the uppermost part of normal mucosa, but the morphology of these positive cells was typical apoptotic bodies (Fig. 5). In the other area of the tubules of adenoma corresponding to the normal crypt, the TUNEL positive cells were sporadic and took the form of apoptotic bodies (Fig. 5). The average TUNEL and Ki-67 labeling indices were 1.61% and 17.0%, respectively (Table I).

Expression of bcl-2 and p53 Protein in Colorectal Tumors

In normal mucosa, expression of bcl-2 was observed in the cells at the bottom of the crypts, but was absent in the superficial portion of the mucosa (Fig. 6). In contrast, the expression of p53 was not observed in normal mucosa. Fifty-two of 63 adenomas (82.5%) and 31 of 49 carcinomas (63.3%) showed the expression of bcl-2. Sixteen of 63 adenomas (25.4%) and 19 of 49 carcinomas (38.8%) were positive for nuclear p53 immunostaining. In adenomas and carcinomas, bcl-2 and p53 positive cells showed no specific topographical features. There was no observable relationship between the distribution of the TUNEL positive cells and that of the bcl-2 or p53 positive cells.

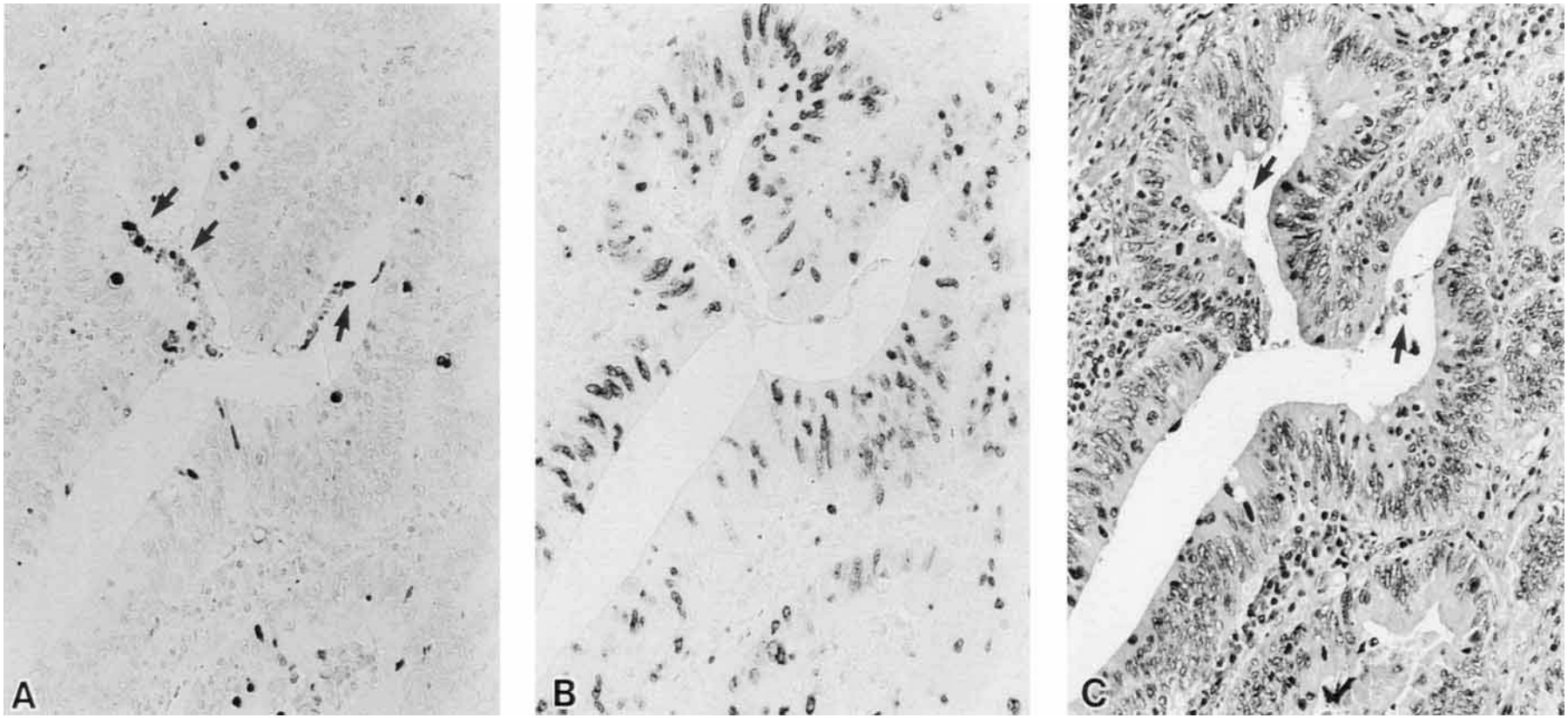

Fig. 1. Serial sections of carcinoma. TUNEL positive cells **(A)** and Ki-67 positive cells **(B)** are randomly distributed. TUNEL positive cells in cancer tubules (arrow in A) are indistinguishable from degenerated cells (arrow in **C,** H/E staining). Original magnification: A–C ×150

Quantitative Observation of Tumors

TLI was significantly higher in adenomas than in normal colorectal mucosa. Both TLI and KLI were significantly higher in carcinomas than in adenomas (TLI: 1.61 + 1.01% compared with 2.83 + 1.79%; KLI: 17.0 + 3.7% compared with 33.1 + 6.7%), but there was no significant difference in TLI/KLI (Table I). There was a positive correlation between TLI and KLI in carcinomas, but not in adenomas (Table II). TLI and TLI/KLI were significantly higher in the adenomas of small size (<6 mm) and/or of low and middle grade atypia than those of large size and/or of high grade atypia (Fig. 7). KLI was higher in the group of carcinoma in adenoma than in that of adenomas of severe grade atypia. No significant difference was noted in TLI and TLI/KLI of the carcinomas classified on the basis of Dukes' stage, tumor size, histological differentiation, and the lymph node metastasis, and no significant correlation was observed between TLI and the expression of the protein product of bcl-2 gene in either adenomas or carcinomas. Moreover, there was no significant correlation between TLI and the expression of p53 in both adenomas and carcinomas.

DISCUSSION

In this study, TUNEL was applied to identify apoptotic cells in colorectal tumors. TUNEL staining detects any kinds of DNA fragmentation in situ [14], including even necrotic cells with random DNA fragmentation [15]. To avoid this problem, the TUNEL positive cells detached from the tumor epithelium were not evaluated (e.g., cells in the lumen of the tumor tubules). Serial morphological changes of tumor cells were also observed from the normal-looking cancer cells into the apoptotic bodies in mirror sections stained by TUNEL and H/E. The evidences obtained from this study support the previous reports that DNA fragmentation precedes the morphological change [16] and that the cleavage of DNA to 300-50 kb fragments occurs in the early apoptotic stage of the epithelial cells [17].

The ratio of TUNEL labeling index to Ki-67 labeling index (TLI/KLI), which is the ratio of apoptotic cells to proliferating tumor cells, means the relative susceptibility of the tumor to apoptosis. The ratio was significantly higher in the adenomas of small size (<6 mm) and/or of low and middle grade atypia than those of large size and/or of high grade atypia. This indicates that the reduction in apoptosis compared with cell proliferation occurs in early stage polyps, and the susceptibility to apoptosis does not change between late stage polyps and early stage carcinomas. KLI of the carcinomas was significantly higher than in adenomas. Therefore, TLI/KLI were not different significantly between adenomas and carcinomas. No significant difference was noted in TLI and TLI/KLI of the carcinomas classified on the basis of Dukes' stage and tumor size. It was concluded that susceptibility to apoptosis decreases in the early phase of the colorectal tumor genesis and does not change in its late phase or the colorectal cancer progression.

Some 82.5% of adenomas expressed the bcl-2 protein and positive cells were also observed at the bottom of normal crypts. This indicates that activation of bcl-2 oncogene plays a critical role in the genesis of colorectal adenomas. The bcl-2 proto-oncogene is a known inhibitor of apoptosis, but its function remains unknown. The present study failed to reveal any significant correlation between TLI and the expression of the bcl-2. Although most adenomas more extensively expressed the gene product of bcl-2 than normal mucosa, the incidence of apoptosis in adenomas was significantly greater than in normal mucosa. It remained unclear whether the inhibition of apoptosis by activation of bcl-2 proto-oncogene had a direct effect on the genesis of colorectal adenomas.

Bronner et al. [18] showed that abnormality of the bcl-2 proto-oncogene was an early event in the gastrointestinal tumor progression. They suggested the abnormality allowed cells to accumulate the genetic and environmental influences necessary for tumor progression, because the product of bcl-2 proto-oncogene was a 26-kd protein that blocked apoptosis and elongated cell life [19–22]. Allan compared apoptotic cells with mitotic and thymidine labeled cells in human breast tumors [23]. They concluded that the reduction in the ratio of apoptosis to mitosis occurring in the normal epithelium increased the risk of development of carcinoma. These results suggest that the reduction of apoptosis is an important event in the progression of some human solid tumors occurring in the early stage of tumor genesis.

Symmonds showed that p53-dependent apoptosis, occurring in response to oncogenic events, was a critical regulator of tumorigenesis [24]. Merritt [25] suggested p53-dependent apoptosis deleted the DNA-damaged cells with carcinogenic potential in the gastrointestinal tract. In the present study, no significant difference was found in TLI between the groups classified on the basis of the immunoreactivity of the anti-p53 antibody. Colorectal cancer results from a series of genetic alterations and the mutation of p53 gene occurs in the late phase of the carcinogenesis [13]. As noted, TLI reduced in the early phase of the carcinogenesis. The reduction of the apoptosis, however, was not accompanied by the alteration of p53 gene in our study. Thus it was not confirmed that p53-dependent apoptosis played an important role in the progression of colorectal carcinoma.

Two groups of cells were observed in the double-stained specimens: (1) cells positive with TUNEL alone, and (2) cells positive with both TUNEL and Ki-67. Ki-67 antibody detects cells in all phases of the cell cycle except those in G0 phase (resting, nonproliferating cells)

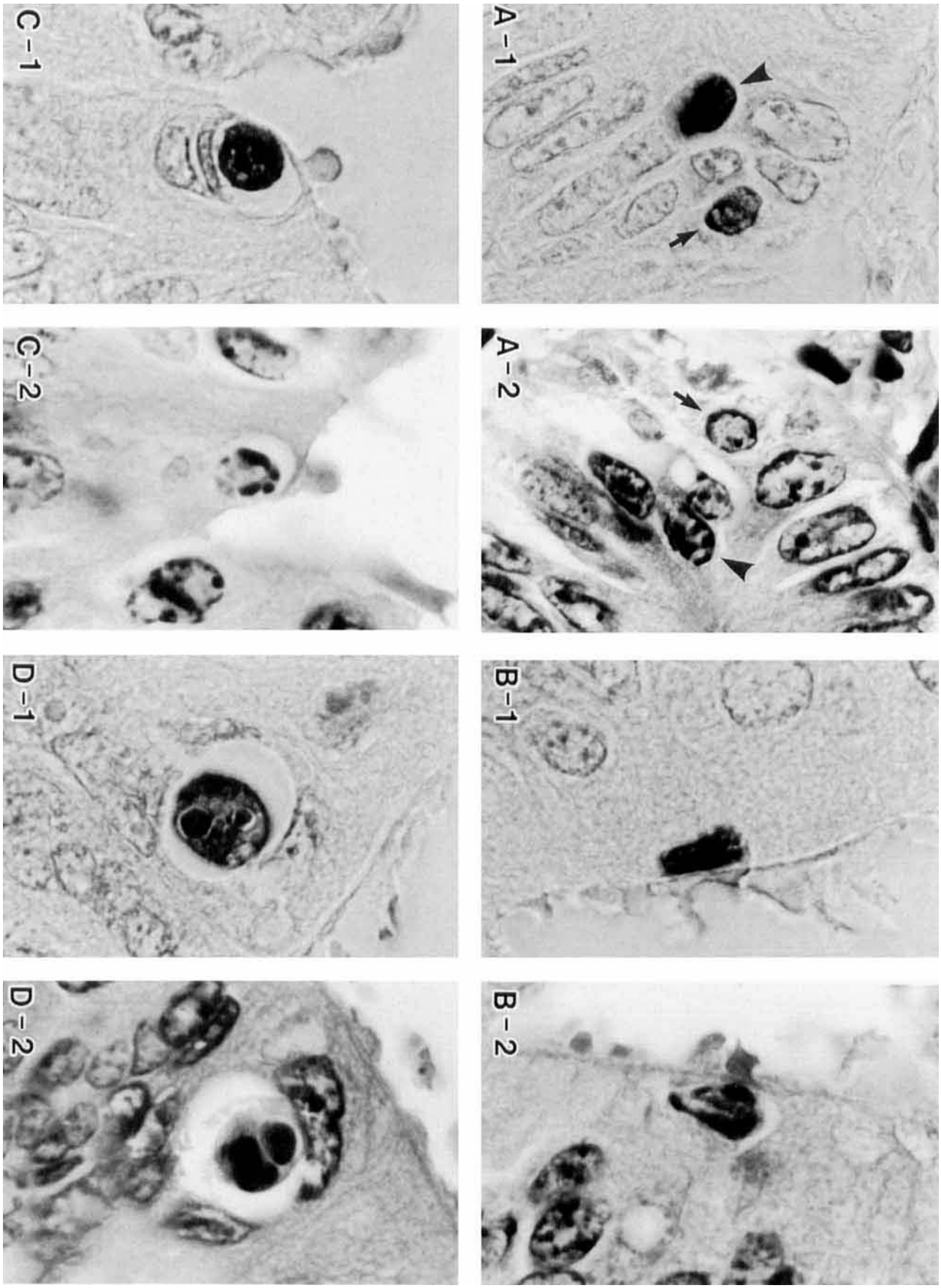

Fig. 2. Legend appears on page 103.

TABLE I. Average of TUNEL and Ki-67 Labeling Indices and Ratio of the Indices*

	Normal mucosa (n = 10)	Adenoma (n = 63)	Carcinoma (n = 49)	*P* value
TUNEL labeling index (TLI) (%)	<0.5	1.61 ± 1.01	2.83 ± 1.79	$P < 0.05$
Ki-67 labeling index (KLI) (%)	15.7 ± 1.9	17.0 ± 3.7	33.1 ± 6.7	$P < 0.05$
TLI/KLI	—	0.097 ± 0.008	0.083 ± 0.006	Not significant

*TLI = TUNEL labeling index; KLI = Ki-67 labeling index.

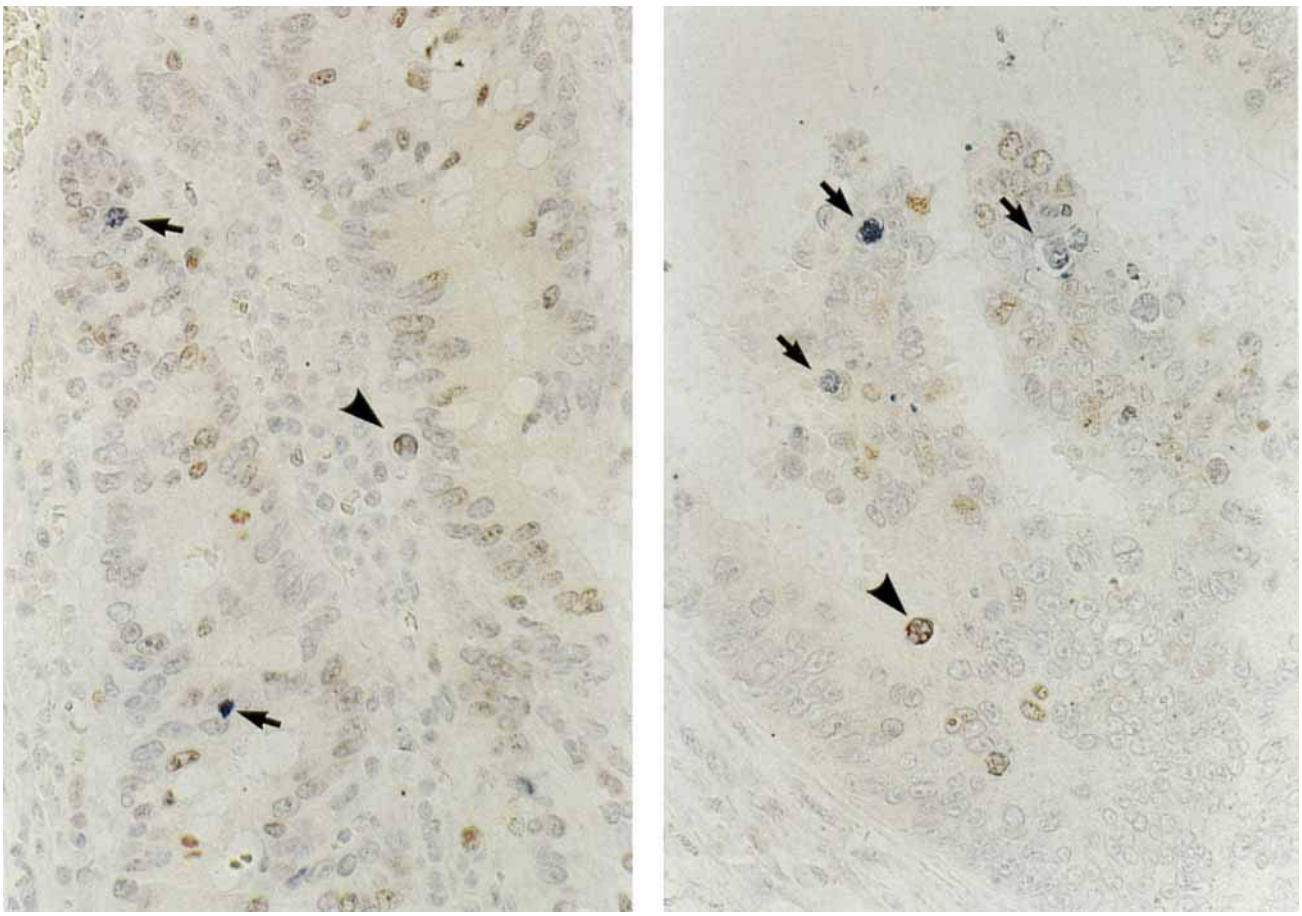

Fig. 3. Double staining consisting of TUNEL and Ki-67 immunostaining of carcinoma. TUNEL positive cells (blue) intermingle with Ki-67 positive cells (red). Apoptotic cells positive with TUNEL alone (arrow) and those positive with both TUNEL and Ki-67 (arrowhead). Original magnification ×200.

Fig. 2. Mirror sections of carcinoma. Each pair are stained by TUNEL and H/E (1: TUNEL, 2: H/E staining). **A.** One TUNEL positive cell shows a normal-looking appearance (arrow) and the other has the slightly condensed nucleus (arrow head). **B.** The TUNEL positive cell partly separated from neighboring cells, because the cytoplasm shrinks. The chromatin condenses under the nuclear membrane. **C.** The TUNEL positive cell has a condensed nucleus and a "halo." **D:** a typical apoptotic body. Original magnification: A–D ×2000.

[26–28]. This implies that apoptosis occurs spontaneously in both proliferating cells and nonproliferating cells in colon tumors. If the apoptosis can be induced at certain points of the cell cycle, there are at least two apoptotic pathways in colorectal tumors. If not, there is an apoptotic pathway by which apoptosis can be induced in cells at any point in the cell cycle. The relation between the apoptotic pathway and the cell cycle observed in solid tumors is still unknown. It is supposed that the DNA-damaged cells arrest the cell cycle in G1 or G2 phase

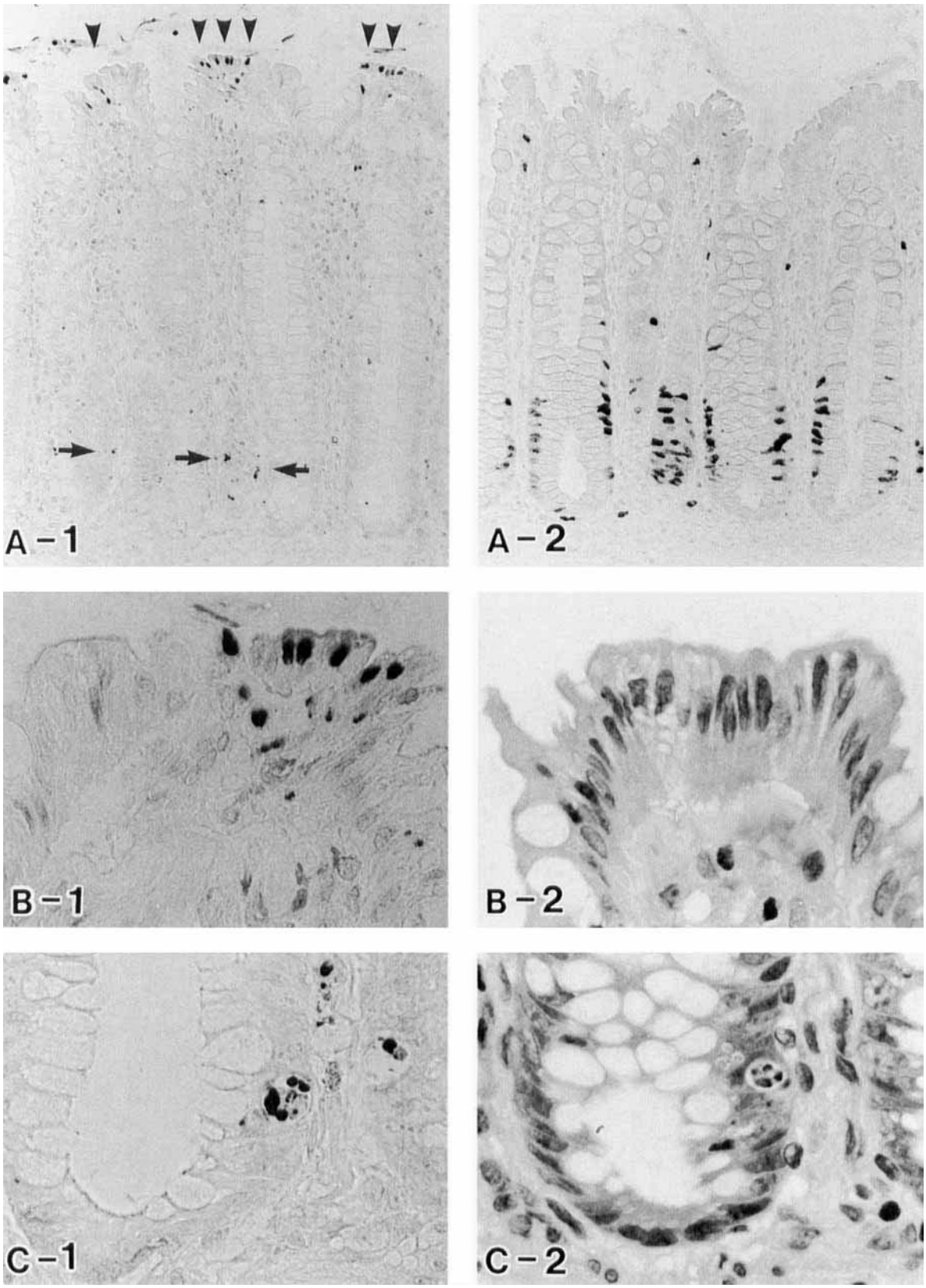

Fig. 4. TUNEL (**A**-1) and Ki-67 immunostaining (**A**-2) of normal mocosa. TUNEL positive cells are seen in upper (arrow head) and lower area (arrow) of the crypt. In the upper crypt area, the positive cells form clusters (**B**-1, TUNEL) and no apoptotic body is observed (**B**-2, H/E staining). In the lower crypt area, the positive cells exist sporadically (**C**-1, TUNEL) and are typical apoptotic bodies (**C**-2, H/E staining). Original magnification: A ×150, B–C ×400.

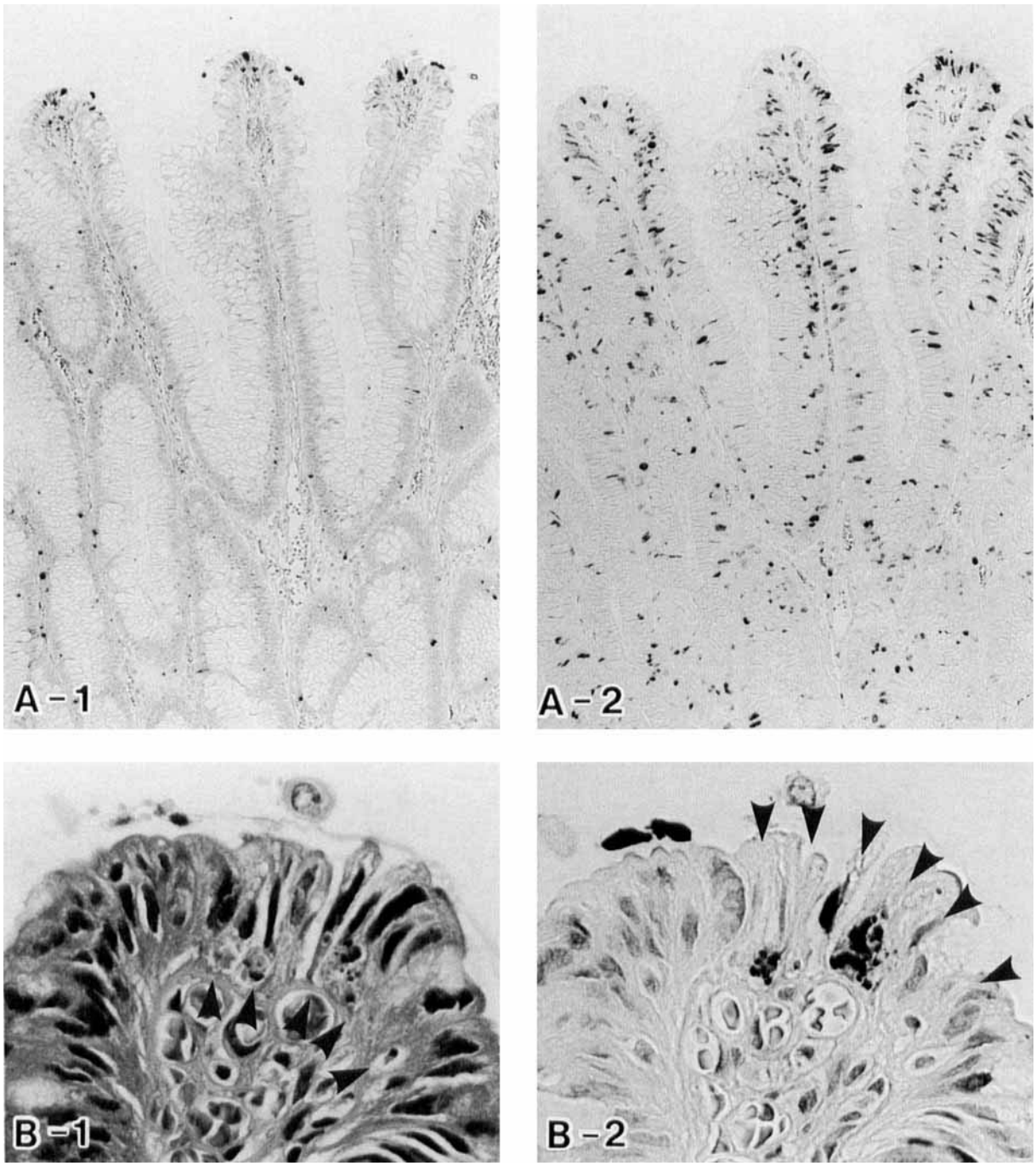

Fig. 5. TUNEL (**A**-1) and Ki-67 immunostaining (**A**-2) of adenoma. In both specimens the positive cells are seen along the whole length of the adenoma-tubules. The greater magnification of the uppermost area (**B**-1: TUNEL, 2: H/E staining). The apoptotic bodies form cluster (arrowhead). Original magnification: A ×100, B ×400.

and deleted through apoptotic pathway if the damage can not be repaired. p53 gene arrests the cell cycle of the DNA-damaged cells in G1 phase and induces apoptosis [29]. However, overexpression of c-myc gene can cause apoptosis in cells at any point of the cell cycle, even in resting cells, without arresting the cell cycle [9]. To understand the molecular mechanism of apoptotic pathways in colorectal tumors, further research is needed.

In the normal mucosa, TUNEL positive cells were observed in the uppermost part and lower half of the crypt. The positive cells in the lower half were apparent apoptotic cells in morphology and very few, but they

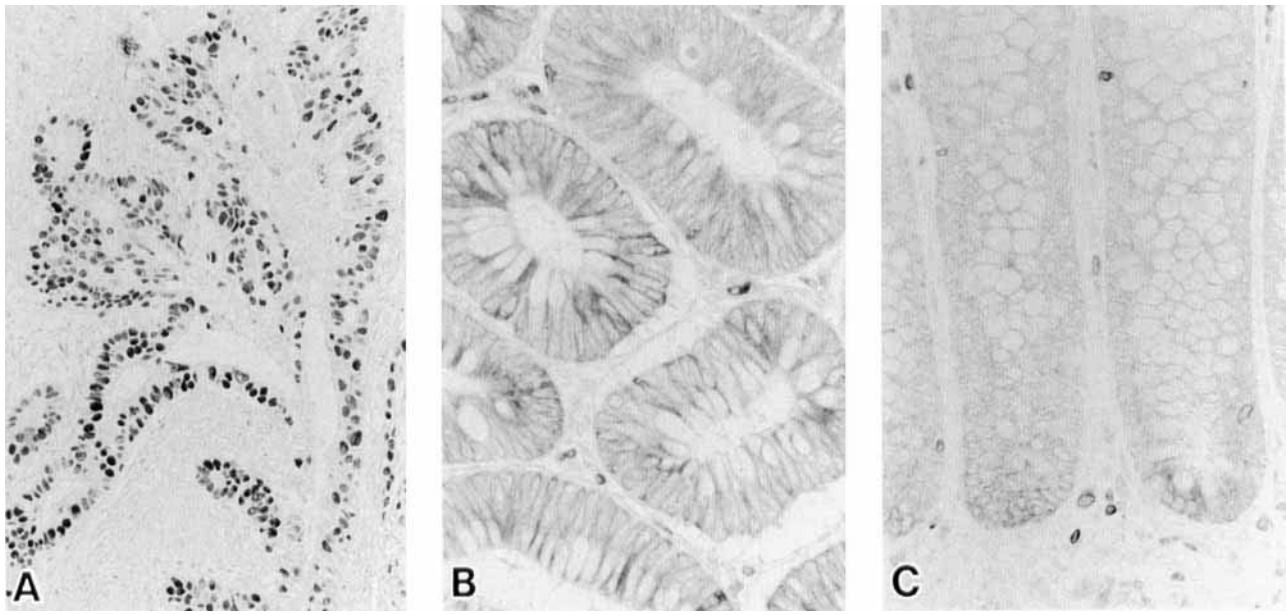

Fig. 6. Nuclear expression of p53 in carcinoma (**A**). Membrane staining for bcl-2 in carcinoma (**B**) and at the bottom of normal crypt (**C**). Original magnification: A–B ×150, C ×200.

TABLE II. Correlation Between TLI and KLI in Adenomas and Carcinomas*

	Pearson's correlation		
	n	coefficient (r)	*P* value
Adenomas	63	0.11	0.39
Carcinomas	49	0.56	<0.0001

*TLI = TUNEL labeling index; KLI = Ki-67 labeling index.

distributed almost periodically. The apoptosis observed in this area is supposed to control the number of crypt cells by removal of excess cells and/or removal of cells containing random genetic defects [30]. No apoptotic cells, however, were found at the top of the crypts. Even ultrastructural observation failed to reveal any apoptotic changes of the epithelial cells there [31]. Thus the cell death seen at the end stage in the terminal differentiation pathway of epithelial cells in colon mucosa may be different from so-called apoptosis. Ki-67 positive cells, in contrast, existed mostly in the lower half of the normal crypt. This would imply the hierarchical structure of large intestine, according to which the epithelial cells proliferate at the bottom of the crypt; some of the proliferated cells are lost there through apoptosis; the surviving cells migrate up and finally shed into the gut lumen, causing DNA fragmentation in their nuclei.

TUNEL positive cells were ubiquitous in the tubular structures of adenomas accompanied by an expanded proliferation zone. However, the staining pattern was different in the superficial and the deep areas of adenomas. In the uppermost part of the tubules corresponding to a normal crypt, TUNEL positive cell distribution was like that in the same part of normal mucosa; in the lower part of the tubules corresponding to the normal crypt, the positive cells distributed as in carcinomas. Presumably, in adenomas the hierarchical structure remains intact in terms of cell position on the surface area.

The percentage of spontaneous apoptosis observed in colon tumors was not so high: 2.83 ± 1.79% in the carcinomas and 1.61 ± 1.01% in the adenomas. However, apoptosis is a rapid process, and the phenotype is transient [30,32]. Even a low level of ~3% of apoptotic cells can result in tissue regression of 25% over several days if not balanced by proliferation [33]. This implies that tumors are losing numerous component cells continuously through apoptosis in their growth process. Although unlimited cell proliferation is one of the peculiar characteristics of tumors, many proliferated cells disappear and do not contribute to tumor growth.

Intensive study on the tumor cell growth has resulted in the concept of the tumor "cell loss," which is derived from the calculation of the fractional difference between the potential tumor doubling time, measured by its cell production rate directly, and the actual tumor doubling time. In colorectal carcinomas, >80% of the proliferating cells disappear from the tissues as "cell loss" [34]. Tumor cell loss has been supposed to occur through exfoliation, differentiation, cell migration, or cell death [35]. In colorectal carcinoma, the tumor cell loss, except apoptosis, is mainly observed on the surface of the lesion, e.g.,

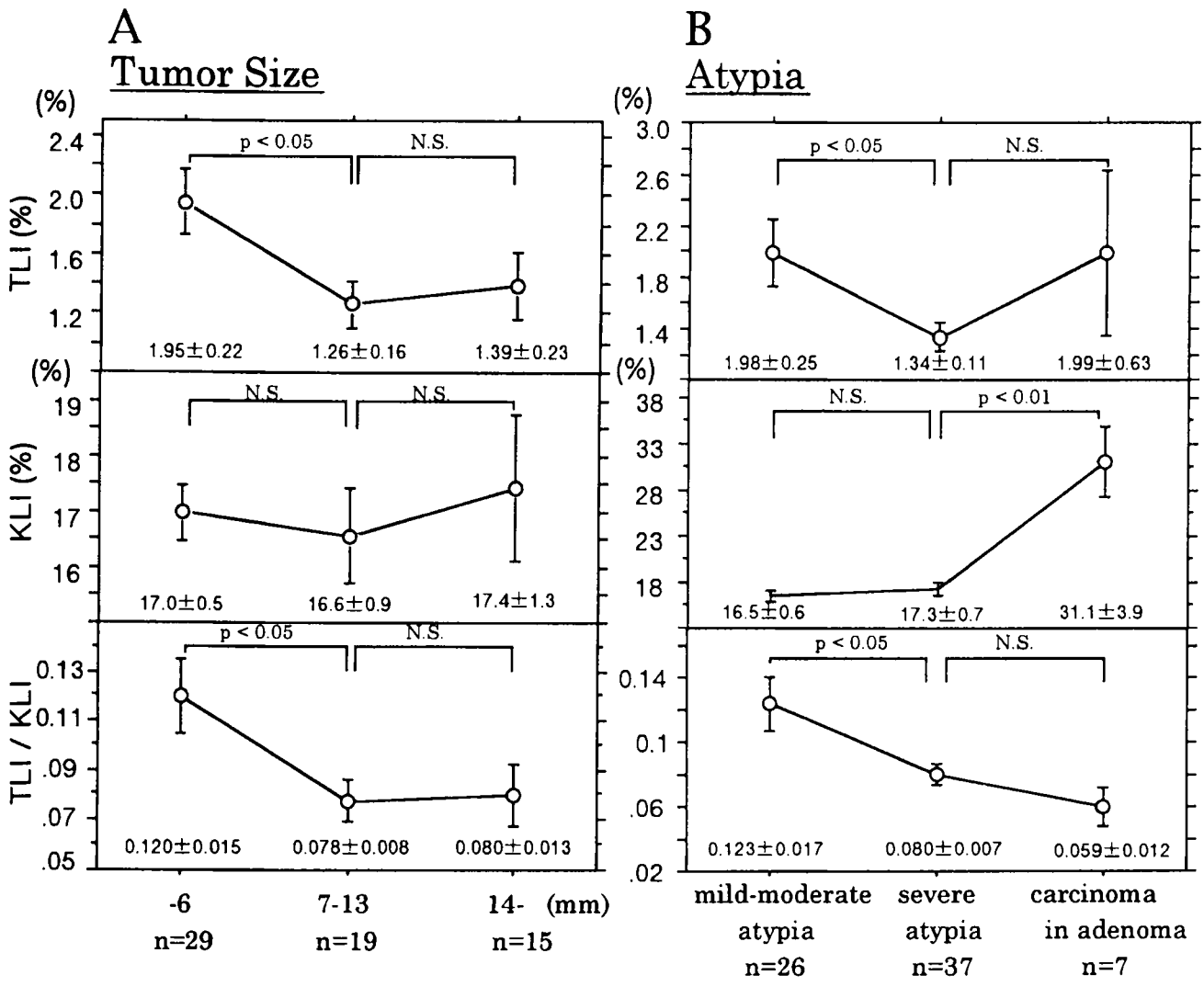

Fig. 7. The indices of the adenomas classified with tumor size (**A**) and atypia (**B**). TLI: TUNEL labeling index, KLI: Ki-67 labeling index.

exfoliation and necrosis occurring at the bottom of the ulcer. The mode of cell loss would appear to explain only part of the "cell loss," because colorectal cancer loses tissue polarity, and no system of transporting cells to the surface was observed like that found in the hierarchical structure of normal colon mucosa. In contrast, everywhere the tumor apoptosis was observed mingling with the proliferating cells, and this mode of cell loss can dispose of numerous tumor cells. Thus apoptosis is a major factor in the "cell loss" of colorectal carcinomas. Apoptosis can define the overall growth rate of the tumors because a tumor is a lesion that arises from the imbalance between cell proliferation and cell loss.

Although an attempt was made to find clinicopathological features that affect TLI in carcinomas, such as Dukes' stage, tumor size, histological differentiation, and the presence of lymph node metastasis, only KLI had a significant correlation with TLI. This correlation implies that the more the tumor cells proliferate, the more the tumor loses cells through apoptosis. In actively proliferating tumors, the mesenchymal environment become worse, and the tumor cells compete for the factors or the signals needed to induce proliferation. Recently, some researchers showed that the deprivation of these factors or signals, such as oxygen, nutrients, several kinds of growth factors and hormones, and intercellular signal, could induce apoptosis [35–37]. The changes accompanying deterioration of the microenvironment are presumed to be one of the major stimuli that induce apoptosis in colorectal carcinomas. There are two reasons why no significant correlation was found between TLI and KLI in adenomas: First, colorectal adenomas reduce KLI, but leave TLI unchanged in the early stage of progression. Second, the mesenchymal environments of adenomas may not deteri-

orate as with carcinomas, because the hierarchical structure of cells remains in the surface area of the adenomas from which many cells are lost.

ACKNOWLEDGMENT

The author is grateful to Prof. Gen Itoh for his advice throughout the study and for his criticism in the preparation of the manuscript.

REFERENCES

1. Kerr JFR, Searle J, Harmons BV: Apoptosis. In Potten CS (ed): "Perspectives on Mammalian Cell Death." Oxford: Oxford Unviersity Press, 1987, p 93–128.
2. Potten CS: Extreme sensitivity of some intestinal crypt cells to x and g-irradiation. Nature 269:518–521, 1977.
3. Allen DJ, Harmon BV, Kerr JFR: Cell death in spermatogenesis. In Potten CS (ed): "Perspectives on Mammalian Cell Death." Oxford: Oxford University Press, 1987, p 229–259.
4. Kerr JFR, Winterford CM, Harmon BV: Apoptosis: Its significance in cancer and cancer therapy. Cancer 73:2013–2026, 1994.
5. Williams GT: Programmed cell death: Apoptosis and oncogenesis. Cell 65:1097–1098, 1991.
6. Oren M: The involvement of oncogenes and tumor suppressor genes in the control of apoptosis. Cancer Metastasis Rev 11:141–148, 1992.
7. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, et al.: Lymphoproliferative disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 356:314–317, 1992.
8. Shaw P, Bovey R, Tardy S, Sahli R, et al.: Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc Natl Acad Sci USA 89:4495–4499, 1992.
9. Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, et al.: Induction of apoptosis in fibroblasts by c-myc protein. Cell 69:119–128, 1992.
10. Vaux DL, Croy S, Adams JM: Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 335:440–442, 1988.
11. Arends MJ, Morris RG, Wyllie AH: Apoptosis—The role of the endonuclease. Am J Pathol 136:593–608, 1990.
12. Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of Programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501, 1992.
13. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, et al.: Genetic alteration during colorectal tumore development. N Engl J Med 319:525–532, 1988.
14. Asari B, Coates PJ, Greenstein BD, Hall PA: In situ end-labelling detects DNA strand breakes in apoptosis and other physiological states. J Pathol 170:1–8, 1993.
15. Gold R, Schmied M, Giegerich G, Breitschopf H, et al.: Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques. Labo Invest 71:219–225, 1994.
16. Gorczyca W, Gong J, Darzynkiewicz Z: Detection of DNA strand Breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. Cancer Res 53:1945–1951, 1993.
17. Oberhammer F, Wilson JW, Dive C, Morris ID, et al.: Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or the absence of internucleosomal fragmentation. EMBO J 12:3679–3684, 1993.
18. Bronner MP, Culin C, Reed JC, Furth EE: The bcl-2 proto-oncogene and the gastrointestinal epithelial tumor progression model. Am J Pathol 146:20–26, 1995.
19. Hockenbery DM, Nunez G, Milliman C, Schreiber RD, et al.: Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 384:334–336, 1990.
20. Korsmeyer SJ: Bcl-2 initiates a new category of oncogene: Regurator of cell death. Blood 80:879–886, 1992.
21. Vaux DL: Toward an understanding of the molecular mechanisms of physiological cell death. Proc Natl Acad Sci USA 90:786–789, 1993.
22. Reed JC: Bcl-2 and the regulation of programmed cell death. J Cell Biol 124:1–6, 1993.
23. Allen DJ, Howell A, Roberts SA, Williams GT, et al.: Reduction in apoptosis relative to mitosis in histologically normal epithelium accompanies fibrocystic change and carcinoma of the premenopausal human breast. J Pathol 167:25–32, 1992.
24. Symmonds H, Krall L, Remington L, Seanz-Robles M, et al.: p53-dependent apoptosis suppresses tumor growth and progression in vivo. Cell 78:703–711, 1994.
25. Merritt AJ, Potten CS, Kemp CJ, Hickman JA, et al.: The role of p53 in spontanious and radiation-induced apoptosis in the gastrointestinal tract of normal and p53-deficient mice. Cancer Res 54:614–617, 1994.
26. Gerdes J, Lemke H, Baisch H, Wacker H, et al.: Cell cycle analysis of proliferation associated with nuclear antigens defined by a monoclonal antibody Ki-67. J Immunol 133:1710–1715, 1984.
27. Gerdes J, Dallenbach F, Lennert K: Growth fractions in malignant non-Hodgkin's lymphoma (NHL) as determined in situ with the monoclonal antibody Ki-67. Haematol Oncol 2:365–371, 1984.
28. Gerdes J, Lelle RJ, Pichartz H, Heidenreich W, et al.: Growth fractions in breast cancer determined in situ with monoclonal antibody Ki-67. J Clin Pathol 39:977–980, 1986.
29. Yonishi-Rouach E, Resnitzky D, Lotem J: Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature 352:345–347, 1991.
30. Potten CS: The significance of spontaneous and induced apoptosis in the gastrointestinal tract of mice. Cancer Metastasis Rev 11:179–195, 1992.
31. Potten CS, Allen TD: Ultrastructure of cell loss in intestinal mucosa. J Ultrastructure Res 60:272–277, 1977.
32. Kerr JFR, Wyllie AH, Currie AR: Apoptosis: a basic biological phenomenon with-ranging implications in tissue kinetics. Br J Cancer 26:239–257, 1972.
33. Bursch W, Paffe S, Putz B, Barthel G, et al.: Determination of the length of histological stages of apoptosis in normal liner and in altered hepatic foci of rats. Carcinogenesis 11:847–853, 1990.
34. Steel GC (eds): "Growth Kinetics of Tumor." Oxford: Clarendon Press, 1977.
35. Arends MJ, Wyllie AH: Apoptosis: Mechanism and roles in pathology. International Review of Experimental Pathology 32:223–254, 1991.
36. Mayer M, Noble M: N-Acetyle-L-cysteine is a pluripotent protector against cell death and enhancer of trophic factor-mediated cell survival in vitro. Proc Natl Acad Sci USA 91:7496–7500, 1994.
37. Bate RC, Buret Andre, van Helden DF, Horton MA, et al.: Apoptosis induced by inhibition of intercellular contact. J Cell Biol 125:403–415, 1994.